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13. ABSTRACT *(Maximum 200 Words)*

Organization of the actin cytoskeleton is crucial for a diverse set of cell functions including cell division, intracellular signaling, cell shape, and motility. Cancerous cells demonstrate changes in cell adhesion and growth consistent with altered regulation of the actin cytoskeleton. Our research has focused on the regulation of protein phosphorylation, specifically signals mediated by protein phosphatase 1 (PP1), a serine/threonine phosphatase, in these processes. Current work is focused on a class of regulatory subunits, neurabin I and neurabin II, that direct PP1 to the actin cytoskeleton.

Neurabin I and neurabin II were cloned from rat tissue either as PP1 or F-actin binding proteins and contain multiple domains. We have cloned homologs of these proteins from *Xenopus laevis* and *C. elegans*. The high degree of structural homology from worms to mammals in specific domains, such as the PP1 binding domain and PDZ domain, begin to highlight regions most likely to be important for the *in vivo* function of these proteins in these diverse species.

GFP-tagged neurabins were expressed in several mammalian cells and established the requirement for an N-terminal actin-binding domain in the localization of neurabins to the actin cytoskeleton. Expression of GFP-neurabin I lacking C-terminal sequences, which include a SAM (sterile alpha motif) and coiled-coil domains, caused cells to develop extensive surface projections or filopodia. Substitution of the $KIKF^{460}$ sequence, which represents a PP1-binding site conserved in many PP1 regulators, with alanines abolished PP1 binding and the mutant GFP-neurabin I while still localizing to the actin cytoskeleton failed to induce filopodia. The ability of GFP-neurabin I to induce filopodia was also inhibited by treatment of cells with the cell-permeable phosphatase inhibitors, okadaic acid or calyculin A, at concentrations that suppressed PP1 activity. These studies suggested that PP1 recruitment and activity was essential for neurabin I-mediated changes in cell morphology. Thus, we believe that the neurabin I-bound PP1 transduces critical signals required for actin reorganization that regulates filopodia formation in mammalian cells.

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FOREWORD

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Introduction

Organization of the actin cytoskeleton is crucial for a diverse set of cell functions including cell division, intracellular signaling, cell shape, and motility. Cancerous cells demonstrate changes in cell adhesion and growth consistent with altered regulation of the actin cytoskeleton. Our research has focused on the regulation of protein phosphorylation, specifically signals mediated by protein phosphatase 1 (PP1), a serine/threonine phosphatase, in these processes. Current work is focused on a class of regulatory subunits, neurabin I and neurabin II, that direct PP1 to the actin cytoskeleton.

Annual Summary

The role of protein phosphorylation in many cell processes is well established. Much of the focus of this research, however, has been on protein kinases, while the importance of protein phosphatases has not been addressed to the same extent. There is growing evidence that protein dephosphorylation is as important as protein phosphorylation in several cellular events such as proliferation, migration, and cell shape. My work over the last year has focused on neurabins, a family of cytoskeletal proteins, which we identified in a protein expression screen of a Xenopus cDNA library using protein phosphatase 1 (PP1) as a probe.

PP1 is a serine/threonine phosphatase which has been implicated in various cellular events, many of which involve hormonal and second messenger signals. However, PP1 itself is not directly modified in response to these signals. A major question in the field has been, how then is PP1 activity controlled by the cell? It now appears that the catalytic subunit of PP1 is controlled by its interaction with various regulatory subunits. Over 40 regulatory/targeting subunits have now been identified and these proteins affect the localization and possibly substrate specificity of the PP1 catalytic subunit. Unlike the catalytic subunit, many of these targeting subunits are modulated by second messenger signaling.

The neurabin family of proteins, neurabin I (NrbI) and neurabin II/spinophilin (NrbII), were first identified as F-actin binding proteins [1, 2] and soon after found to interact with PP1 as well [3]. NrbI is localized exclusively to brain while NrbII is ubiquitously expressed. Both of these proteins contain a PDZ domain and a coiled-coil domain, which are thought to mediate protein-protein interactions. They also contain a consensus PP1 binding domain, RVXF, which is found in many PP1 interacting proteins [4]. The high degree of homology between NrbI and NrbII among several different

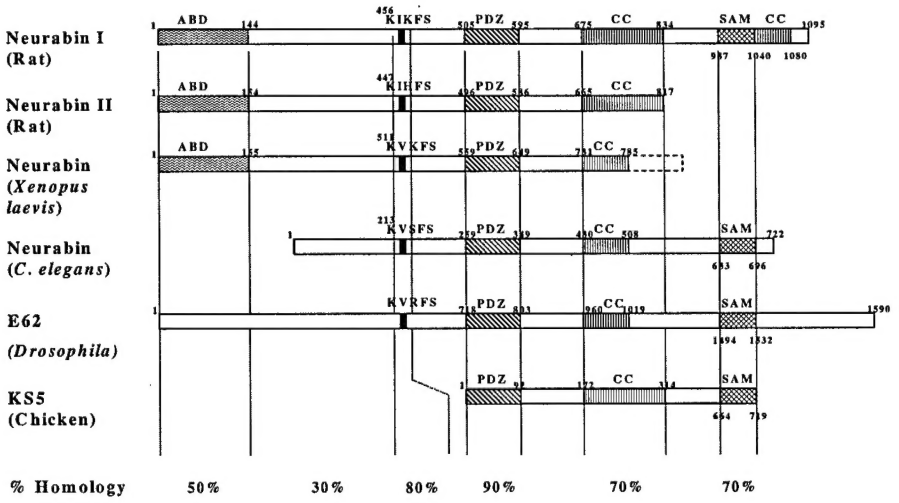

Figure 1 - Homology among different domains of neurabin family members. ABD -actin binding domain, PDZ - PSD95/DLG/ZO-1, CC - Coiled-Coil domain, SAM - sterile alpha motif.

species points to domains which may be important for neurabins' physiological role (Figure 1).

For the past year, we have focused our attention on the actin binding domain, PP1

binding domain, PDZ domain, and coiled coil domains in an attempt to determine the physiological function of neurabin I. Previously, we had made several green fluorescent protein (GFP) tagged constructs of neurabin I to attempt to isolate the function of some of these domains. We showed that HEK293 cells expressing GFP-NrbI (1-552) exhibited an altered morphology when compared to cells expressing GFP alone or full-length GFP-NrbI (1-1095). These cells had numerous filopodia-like projections to which the GFP-NrbI (1-552) protein localized. In order to determine the nature of these projections we transiently transfected HEK293 cells with either GFP-NrbI (1-1095) or GFP-NrbI (1-552) and performed live video microscopy (Figure 2A). Time lapse micrographs of these cells confirm that the projections seen in GFP-NrbI (1-552) expressing cells are filopodia and not retraction fibers. These data also confirm that GFP-NrbI (1-1095) expressing cells do not contain filopodia. Thus, the coiled-coil domain of neurabin I, which is deleted in the GFP-NrbI (1-552) construct appears to inhibit filopodia formation in the context of the full-length protein. There is some evidence that coiled-coil and SAM domains can mediate dimerization in general [5, 6] and specifically in neurabin I [7], we therefore looked to see if the coiled-coil domain of neurabin I could bind to neurabin I or II from rat brain extracts (Figure 2B). His-tagged NrbI proteins containing the coiled coil domain bind to rat brain NrbI and NrbII, while NrbI proteins without the coiled-coil domain are not able to bind to rat brain NrbI or NrbII. These data suggest that the dimerization of neurabin I inhibits its ability to induce filopodia and that because GFP-NrbI (1-552) has no coiled-coil domain, it is monomeric and able to induce filopodia.

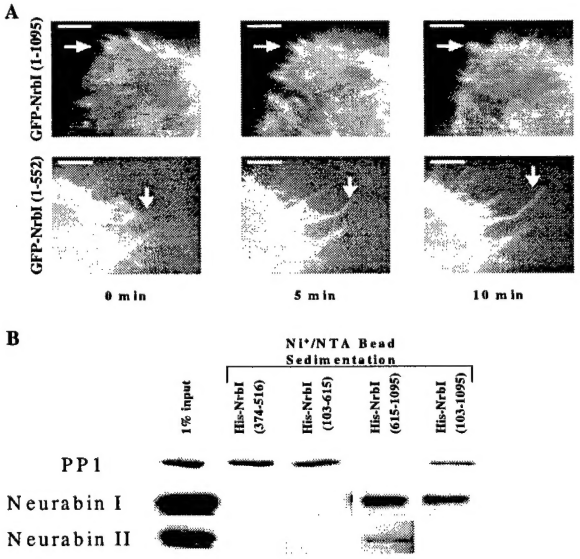

Figure 2 - Role of NrbI Coiled-Coil Domain in Filopodia Formation. Panel A shows time lapse videomicroscopy of HEK293 cells expressing GFP-NrbI (1-1095) or GFP-NrbI (1-552) over a 10 minute time course. Arrows highlight membrane protrusions of interest. Bar = 25 μm. Panel B shows pulldowns from deoxycholate homegenates of rat brain using His-NrbI (374-516), His-NrbI (103-615), His-NrbI (615-1095), and His-NrbI (103-1095). Proteins were run on 10% (w/v) SDS-PAGE and immunoblotted for PP1, NrbI, or NrbII as indicated.

We had previously shown that GFP-NrbI can immunoprecipitate PP1 out of HEK293 cells and that GFP-NrbI (1-552, AAAA), which substituted alanines in place of the KIKF PP1 binding motif, cannot bind to PP1. The anti-GFP immunoprecipitates

were subsequently assayed for PP1 activity using the standard substrate, phosphorylase *a* (Figure 3A). The immune complex PP1 assays established, for the first time, that cellular NrbI-PP1 complexes represented active protein phosphatases. When corrected for equivalent amounts of GFP proteins, GFP-NrbI (1-552) immunoprecipitates consistently contained 50-60% more protein phosphatase activity than other GFP-NrbI proteins. As anticipated, immunoprecipitates containing GFP-NrbI (1-552, AAAA) demonstrated little or no phosphatase activity, as noted with GFP alone. These data suggested that GFP-NrbI (1-552) recruited PP1 more effectively than GFP-NrbI (1-1095) or GFP-NrbI (286-1095) and PP1 binding absolutely required the KIKF sequence in NrbI.

To confirm the identity of GFP-NrbI (1-552)-bound phosphatase, immune complex assays were also performed in the presence of okadaic acid (Figure 3A). Okadaic acid (5 nM), sufficient to inhibit PP2A-like phosphatases, had no effect on the GFP-NrbI (1-552)-bound phosphatase and okadaic acid concentration above 100 nM was needed to inhibit the NrbI-bound phosphatase. NrbI (1-552)-bound phosphatase was fully inhibited by 1 μM okadaic acid, consistent with its identity as PP1 [8].

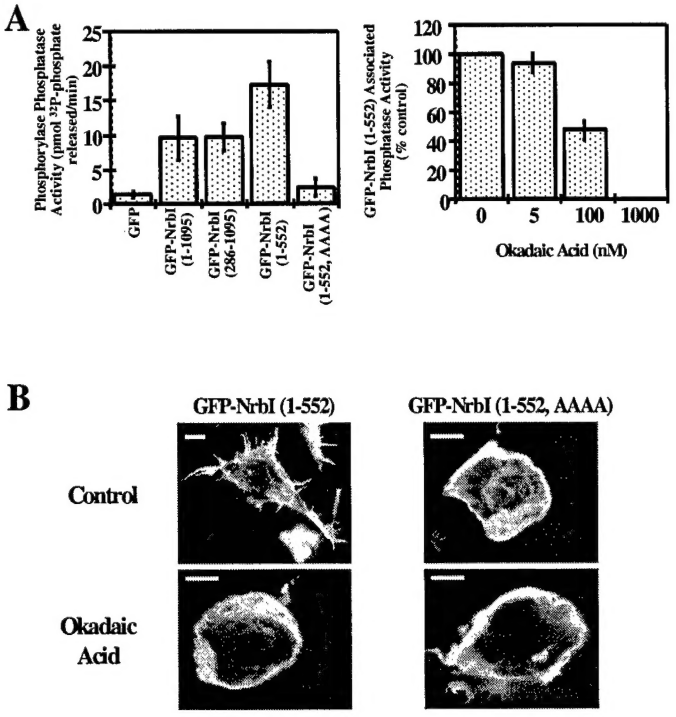

Figure 3 - PP1 is Required for Filopodia Formation. Panel A shows phosphorylase a phosphatase activity associated with GFP-NrbI immunoprecipitates. Left panel shows phosphatase activity represented as ^{32}P-phosphate released by equivalent amounts of immunoprecipitates assessed by immunoblotting with anti-GFP. The data represents the average of three independent experiments performed in duplicate (shown with standard errors bars). Right panel shows the sensitivity of the phosphatase activity associated with GFP-NrbI (1-552) to okadaic acid and represents the average of three independent experiments (shown with standard error bars). Panel B shows HEK293 cells expressing GFP-NrbI (1-552) or GFP-NrbI (1-552, AAAA) treated with okadaic acid (1 μM) for 15 minutes prior to fixation. Scale bar = 25 μm.

While GFP-NrbI (1-552) expression induced extensive filopodia in HEK293 cells, GFP-NrbI (1-552, AAAA), while still localized to the cortical actin cytoskeleton, failed to induce filopodia (Figure 3B). A majority of GFP-NrbI (1-552, AAAA)-expressing cells (71%, n=208) possessed a rounded morphology similar to that seen in cells expressing WT GFP-NrbI (1-1095). Treatment of GFP-NrbI (1-552)-expressing cells with okadaic acid (1 μM) (Figure 3B) or calyculin A (10 nM) (data not shown) to eliminate PP1 activity associated with GFP-NrbI (1-552) resulted in near complete loss of filopodia. Okadaic acid and calyculin A had no effect on the morphology of cells expressing GFP-NrbI (1-552, AAAA) or GFP-NrbI (1-1095). Thus, mutating the PP1-binding motif or inhibiting the activity of NrbI-bound PP1 had similar effects on cell morphology and specifically established the importance of the NrbI (1-552)/PP1 complex in the formation of filopodia.

p70S6K functions in opposition to PP1 to inhibit actin-based events, such as neurite outgrowth [9] and cytokinesis [10] that are activated by PP1. To investigate the

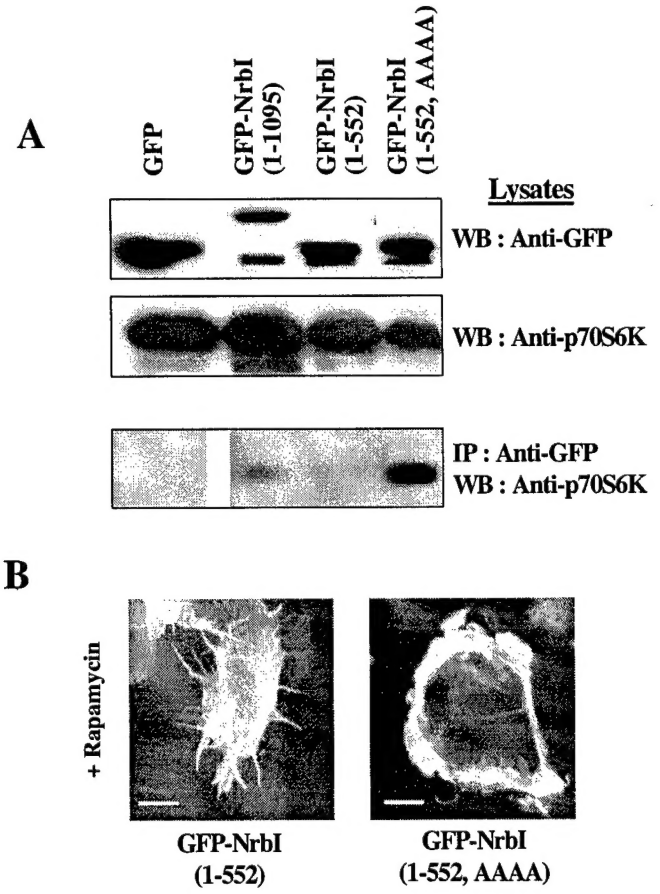

Figure 4 - PP1 Competes for Neurabin I Binding with p70 Ribosomal S6 Protein Kinase. Panel A – HEK293 cells expressing HA-tagged p70S6K and GFP, GFP-NrbI (1-1095), GFP-NrbI (1-552), or GFP-NrbI (1-552, AAAA) were lysed in RIPA buffer, lysates clarified by centrifugation and GFP-NrbI immunoprecipitated with anti-GFP. Immunoprecipitates were subjected to 10% (w/v) SDS-PAGE and immunoblotted with anti-p70S6K. Panel B shows micrographs of cells expressing GFP-NrbI (1-1095), GFP-NrbI (1-552) or GFP-NrbI (1-552, AAAA) following 20 min treatment with 50 nM rapamycin. Scale bar = 25 μm.

possibility that NrbI assembles a kinase-phosphatase module that regulates actin assembly, we compared the ability of GFP-NrbI proteins to recruit p70S6K by immunoprecipitation. In contrast to the readily observed binding of GFP-NrbI (1-1095) to endogenous PP1 in HEK293 cells, GFP-NrbI (1-1095) failed to show any significant association with endogenous p70S6K (data not shown). However, co-expression of GFP-NrbI with HA-tagged p70S6K established that p70S6K bound WT GFP-NrbI (1-1095) but not GFP (Figure 4A). Compared to WT GFP-NrbI (1-1095), immunoprecipitates containing GFP-NrbI (1-552) consistently contained less HA-tagged p70S6K. This suggested potential competition between PP1 and p70S6K for NrbI binding. Thus, we analyzed p70S6K binding to GFP-NrbI (1-552, AAAA) that fails to bind PP1. GFP-NrbI (1-552, AAAA) showed greatly increased p70S6K binding compared to either GFP-NrbI (1-1095) or GFP-NrbI (1-552), supporting a competition between PP1 and p70S6K for NrbI. This suggested the presence of distinct cellular neurabin/PP1 and neurabin/p70S6K complexes with PP1 being preferred over p70S6K for NrbI binding in cells.

Overexpression of HA-tagged p70S6K had no discernable effect on cell morphology (data not shown) but to exclude the possible role of the NrbI/p70S6K complex in actin reorganization, cells expressing GFP-NrbI (1-1095), GFP-NrbI (1-552) and GFP-NrbI (1-552, AAAA) were treated with 50 nM rapamycin to inhibit p70S6K activity (ref). Rapamycin had no effect on the number or size of filopodia in HEK293 cells expressing GFP-NrbI (1-552) or the morphology of cells expressing GFP-NrbI (1-552, AAAA) (Figure 4B) and the role of the NrbI/p70S6K complex, if any, in actin rearrangement in HEK293 cells is unclear.

In conclusion, our studies demonstrated that the actin-bound neurabin I recruits PP1 activity to regulate cytoskeletal dynamics and cell morphology, inducing filopodia in neurons and non-neuronal cells and thus, shed new light on the role of PP1-catalyzed protein dephosphorylation in cell shape and function. This novel role for PP1 has the potential to shed new light on the role of phosphatases in migration and invasiveness of cancer cells and might eventually lead to new therapies to prevent metastasis.

Key Research Accomplishments

- Cloned *Xenopus* neurabin and showed it is a bona fide PP1 binding protein.
- Established that GFP-NrbI (1-552), missing coiled-coil domain, forms filopodia in HEK293 cells by time-lapse video microscopy in live cells.
- Established that the coiled-coil domain of NrbI alone can heterodimerize with endogenous NrbI and NrbII in pulldowns out of rat brain extracts.
- Showed that there is phosphatase activity associated with GFP-NrbI (1-1095), GFP-NrbI (286-1095), and GFP-NrbI(1-552), but not GFP-NrbI (1-552,AAAA) which has the PP1 binding domain deleted.
- Confirmed that the phosphatase activity associated with GFP-NrbI is PP1.
- Determined that PP1 association with GFP-NrbI (1-552) is required for filopodia formation in HEK293 cells.
- Showed that PP1/NrbI and p70S6K/NrbI represent distinct complexes and so are mutually exclusive.
- Established that p70S6K/NrbI complex does not modulate filopodia in HEK293 cells.

Reportable Outcomes

Oliver, C.J., Terry-Lorenzo, R.T., Elliott, E., Christensen, W.A., Li, S., Brautigan, D.L., Colbran, R.J., and Shenolikar, S. (2001). Neurabin I-bound protein phosphatase-1 induces. Manuscript submitted.

Oliver, C.J., Terry-Lorenzo, R.T., Christensen, W.A., Colbran, R.J., and Shenolikar, S. (2001). Protein Phosphatase-1 Association with the Neuronal Actin-Binding Protein, Neurabin I, Induces Filopodia in Neurons and Non-Neuronal Cells. EMBO Conference on Protein Phosphorylation and Protein Phosphatases.

Terry-Lorenzo, R.T., Inoue, M., Connor, J.H., Haystead, T.A.J., Armbruster, B.N., Gupta, R.P., **Oliver, C.J.** and Shenolikar, S. (2000). Neurofilament-L is a protein phosphatase-1-binding protein associated with neuronal plasma membrane and post-synaptic density. J. Biol. Chem. 275:2439-2446.

Liu, J., Wu, J., **Oliver, C.**, Shenolikar, S. and Brautigan, D.L. (2000). Mutations of the serine phosphorylated in the protein phosphatase-1-binding motif in the skeletal muscle glycogen-targeting subunit. Biochem. J. 346:77-82.

Oliver, C.J., Li, S., Colbran, R.J., and Shenolikar, S. Protein phosphatase-1 - targeting the actin cytoskeleton. (2000) FASEB Summer Research Conference on Protein Phosphatases.

Oliver, C.J. and Shenolikar, S. Protein phosphatase-1 and cytoskeletal organization. (2000) Department of Defense Breast Cancer Program Era of Hope Meeting.

Oliver, C.J. and Shenolikar, S. (1998). Physiologic importance of protein phosphatase inhibitors. Front. Biosci. 3:961-972.

Wu, J., Liu, J., Thompson, I., **Oliver, C.J.**, Shenolikar, S. and Brautigan, D.L. (1998). A conserved domain for glycogen binding in protein phosphatase-1 targeting subunits. FEBS Lett. 439:185-191.

Conclusions

In summary, we have begun to look at the physiological relevance of one member of the neurabin family of proteins, neurabin I. By expressing various GFP-tagged proteins in a heterologous cell line, HEK-293 which do not contain endogenous neurabin I, we have been able to determine several properties of this protein *in vivo*. One, the actin binding domain contained within the first 150 amino acids is absolutely required for interaction of this protein with the actin cytoskeleton. Two, deletion of the PDZ and coiled-coil domains leads to an increase in filopodial formation and localization of the protein to filopodia. Three, the RVXF motif of neurabin I is absolutely required for PP1 binding *in vivo*, since mutation of these residues to alanine abrogates PP1 binding. Four, the recruitment of PP1 to the actin cytoskeleton by GFP-NrbI(1-552) is absolutely required for filopodia formation. Five, binding of PP1 and p70 S6K to neurabin I are mutually exclusive. These studies show that the neurabin I-bound PP1 regulates the formation of filopodia and yields new insights into a mechanism that targets PP1 to the actin cytoskeleton.

References

1. Nakanishi, H., H. Obaishi, A. Satoh, M. Wada, K. Mandai, K. Satoh, H. Nishioka, Y. Matsuura, A. Mizoguchi, and Y. Takai, *Neurabin: a novel neural tissue-specific actin filament-binding protein involved in neurite formation.* J Cell Biol, 1997. **139**(4): p. 951-61.

2. Satoh, A., H. Nakanishi, H. Obaishi, M. Wada, K. Takahashi, K. Satoh, K. Hirao, H. Nishioka, Y. Hata, A. Mizoguchi, and Y. Takai, *Neurabin-II/spinophilin. An actin filament-binding protein with one pdz domain localized at cadherin-based cell-cell adhesion sites.* J Biol Chem, 1998. **273**(6): p. 3470-5.

3. Allen, P.B., C.C. Ouimet, and P. Greengard, *Spinophilin, a novel protein phosphatase 1 binding protein localized to dendritic spines.* Proc Natl Acad Sci U S A, 1997. **94**(18): p. 9956-61.

4. Egloff, M.P., D.F. Johnson, G. Moorhead, P.T. Cohen, P. Cohen, and D. Barford, *Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1.* Embo J, 1997. **16**(8): p. 1876-87.

5. Lupas, A., *Coiled coils: new structures and new functions.* Trends Biochem Sci, 1996. **21**(10): p. 375-82.

6. Stapleton, D., I. Balan, T. Pawson, and F. Sicheri, *The crystal structure of an Eph receptor SAM domain reveals a mechanism for modular dimerization.* Nat Struct Biol, 1999. **6**(1): p. 44-9.

7. MacMillan, L.B., M.A. Bass, N. Cheng, E.F. Howard, M. Tamura, S. Strack, B.E. Wadzinski, and R.J. Colbran, *Brain actin-associated protein phosphatase 1 holoenzymes containing spinophilin, neurabin, and selected catalytic subunit isoforms.* J Biol Chem, 1999. **274**(50): p. 35845-54.

8. Cohen, P., C.F. Holmes, and Y. Tsukitani, *Okadaic acid: a new probe for the study of cellular regulation.* Trends Biochem Sci, 1990. **15**(3): p. 98-102.

9. Parker, E.M., A. Monopoli, E. Ongini, G. Lozza, and C.M. Babij, *Rapamycin, but not FK506 and GPI-1046, increases neurite outgrowth in PC12 cells by inhibiting cell cycle progression.* Neuropharmacology, 2000. **39**(10): p. 1913-9.

10. Sugiyama, H., P. Papst, M. Fujita, E.W. Gelfand, and N. Terada, *Overexpression of wild type p70 S6 kinase interferes with cytokinesis.* Oncogene, 1997. **15**(4): p. 443-52.

Appendix 1

Protein Phosphatase-1 Association with the Neuronal Actin-Binding Protein, Neurabin I, Induces Filopodia in Neurons and Non-Neuronal Cells

Oliver, C.J., Terry-Lorenzo, R.T., Christensen, W.A., Colbran, R.J.*, and Shenolikar, S.

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The organization of actin cytoskeleton is crucial for many cellular functions, including cell division, signal transduction, cell morphology and migration. Our research is directed at elucidating the signals transduced by protein phosphatase-1(PP1), a serine/threonine phosphatase, to organize the actin cytoskeleton and control cell growth and differentiation. Mammalian PP1 exists as a complex of the PP1 catalytic subunit with one or more regulatory proteins. This work is focused on two isoforms of the neuronal actin-binding protein, neurabin I and neurabin II/spinophilin, that target PP1 to the actin cytoskeleton.

Neurabin I and neurabin II, first cloned from rat brain as F-actin and PP1-binding proteins respectively, contain multiple protein interaction domains. We have also cloned the neurabin homologues from *Xenopus laevis* and *Caenorhabditis elegans* and noted particularly striking homologies within the PP1-binding and PDZ domains in neurabins from worms to man. This suggested important roles for the proteins recruited by these domains in the physiological functions of neurabins.

GFP-tagged neurabins were expressed in several mammalian cells and established the requirement for an N-terminal actin-binding domain in the localization of neurabins to the actin cytoskeleton. Expression of GFP-neurabin I lacking C-terminal sequences, which include a SAM (sterile alpha motif) and coiled-coil domains, caused cells to develop extensive surface projections or filopodia. Substitution of the $KIKF^{460}$ sequence, which represents a PP1-binding site conserved in many PP1 regulators, with alanines abolished PP1 binding and the mutant GFP-neurabin I while still localizing to the actin cytoskeleton failed to induce filopodia. The ability of GFP-neurabin I to induce filopodia was also inhibited by treatment of cells with the cell-permeable phosphatase inhibitors, okadaic acid or calyculin A, at concentrations that suppressed PP1 activity. These studies suggested that PP1 recruitment and activity was essential for neurabin I-mediated changes in cell morphology.

The PDZ domain in neurabin I binds the p70 ribosomal S6 protein kinase (p70S6K), which has been implicated in the negative regulation of actin-based processes that include neurite outgrowth and cytokinesis. To examine the potential role for neurabin I as a site for kinase-phosphatase crosstalk that regulates actin reorganization, we undertook immunoprecipitation studies in cells expressing GFP-neurabins and variants of p70S6K. These studies showed that the GFP-neurabins which contained a functional PP1-binding site bound little p70S6K while GFP-neurabin proteins incapable of associating with PP1 recruited higher levels of p70S6K. These studies suggested a potential competition between PP1 and p70S6K for docking at adjacent sites on neurabin I. Overexpression of p70S6K and treatment with rapamycin have thus far failed to elicit morphological alterations in HEK293 cells. Thus, our studies suggest that the neurabin I-bound PP1 transduces critical signals required for actin reorganization that regulates filopodia formation in mammalian cells.